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Method Article**Tooth bioengineering from single cell suspensions****Vassilis Stratoulis^{1,*} and Frederic Michon^{1,2}**

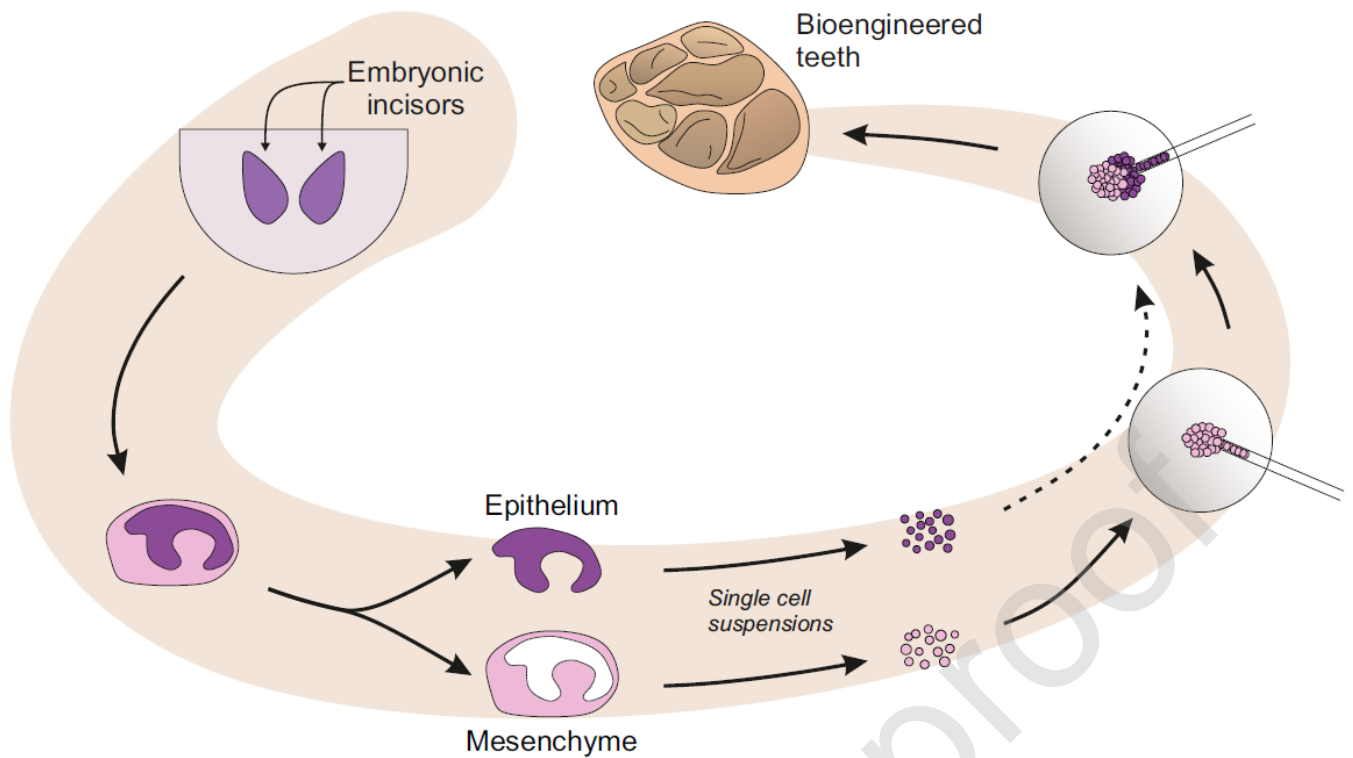
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Graphical abstract



Graphical abstract_Stratoulas & Michon

Abstract

Recent advances in bioengineering and biomaterials, along with knowledge deriving from the fields of developmental biology and stem cell research, have rendered feasible functional replacement of full organs. Here, we describe the methodology for bioengineering a tooth, starting from embryonic epithelial and mesenchymal single cell suspensions. In addition, we describe the subsequent steps of processing this minute structure for use in applications such as histological examination, immunofluorescence and *in situ* hybridisation. This methodology can be used for any minute structure that needs to be used in paraffin blocks.

- Detailed methodology for reproducible and reliable results
- Extra step to ensure single cell populations
- Subsequent minute structure processing for histological analysis

Keywords: tooth, bioengineering, reconstruction, dental implant, epithelium, mesenchyme

Introduction

Tissue engineering combines the fields of bioengineering, biomaterials and molecular biology. It aims in generating fully functional tissues that can replace damaged organs. Two main strategies, in which cells that have the potential to repopulate and regenerate the tissue of interest are used as primary source, are utilized in order to produce engineered tissues. First, scaffolds are used in which cells are seeded *in vitro*. These scaffolds can be either synthetic or natural occurring extracellular matrix (decellularized tissues). This strategy has been used in a variety of tissues such as liver [1], heart [2], trachea [3], oesophagus [4] and skeletal muscle [5] among others. The second strategy is trying to recapitulate organogenesis *in vitro* and it has been used with great success for the generation of ectodermal organs including teeth [6, 7], hair follicles [8], salivary [9] and lacrimal glands [10]. Despite the obvious advantage offered by this strategy in studying dental morphogenesis through the plethora of cells of origin (*e.g.* mouse strains, treated cells, iPS cells), it has not been replicated to the degree anticipated.

The tooth is an ectodermal organ that arise from a primordial structure known as tooth germ. During its development, epithelial-mesenchymal interactions are of pivotal importance [11]. In mice, the tooth germ formation is initiated at embryonic day 10,5 (ED10,5) by signals that arise from the epithelium such as FGF8, BMP4, Shh and Eda. These signals result in the expression of an array of transcription factors in the dental mesenchyme. Next, the mesenchyme condenses around the developing epithelial bud. At ED14, a transient epithelial structure known as the enamel knot appears and functions as the signaling center for subsequent tooth morphogenesis [12].

Materials

The tooth reconstitution protocol described here is based on the one by Nakao *et al.* [6] with modifications. All experiments involving live animals must conform to national and institutional regulations. In the present study, all animal protocols were approved by the Finnish National Board of Animal Experimentation (ESAVI/1284/04.10.07/2016). NMRI and *Sox2-GFP* (kind gift from Fred H. Gage, Salk Institute, CA, USA, reference [13]) mice were used and maintained under 12-h light/dark cycle at 22 °C under standard conditions.

Tooth bioengineering and organ culture

For our experiments, we conventionally use incisors from E14,5 mouse embryos. However, we have also successful used incisors from E15,5 mouse embryos.

Reagents

1. Phosphate-buffered saline (PBS), pH 7.4 (Gibco, cat no. 10010023).
2. Dulbecco's modified Eagle's medium (DMEM), supplemented with GlutaMAX™ (Gibco, cat. no. 61965026).
3. Fetal bovine serum (FBS) (HyClone, cat no. SV30160.02).
4. Penicillin/streptomycin (Gibco, cat no. 15140122).
5. Ascorbic acid (Sigma, cat no. A4544).
6. Optimem (Gibco, cat no. 31985070).
7. Dispase II (Sigma, cat no. D4693).
8. DNase I (Thermo Scientific™, cat no. EN0521, final concentration: 20 U/ml).
9. StemPro™ Accutase™ (Gibco, cat no. A1110501).
10. Cellmatrix Type I-A (Nitta-Gelatin Inc, cat no. 638-00781).

Reagent set up

1. Culture medium (serum-containing): DMEM supplemented with GlutaMAX™, 10% FBS, 1% penicillin/streptomycin, 100 µg/ml ascorbic acid.
2. Culture medium (serum-free): as above, but no FBS.
3. Dispase II. Dilute to 50 U/ml in a buffer containing 10mM NaAc pH 7.5 and 5mM CaAc. Store at 4 °C for up to a month.
4. Prepare Cellmatrix as described in the provider's protocol, namely:
 - a. In 8 volumes of ice-cold Cellmatrix, add 1 volume of 10 x concentrated MEM (without NaHCO₃) and mix by vortexing. Solution will acquire a light yellow colour.
 - b. Add 1 volume of reconstitution and mix by vortexing. The solution will acquire a light pink colour.

Perform steps in a cold 50 ml falcon tube on ice. Prepare Cellmatrix at least 10 min prior to use, or after bubbles have disappeared.

Note: All components are included in the Cellmatrix kit (Cellmatrix Type I-A (Nitta-Gelatin Inc, cat no. 638-00781).

Note: Cellmatrix Type I-A (pH 3) is an acid-soluble Type I collagen derives from porcine tendon. Upon reconstitution, it gives a high strength, transparent gel.

Equipment

1. Glass petri dishes.
2. Laminar flow hood.
3. Dissecting stereomicroscope.
4. Sterilized microdissecting instruments: Scissors, forceps, fine needles.

5. Agitator.
6. Eppendorf tubes.
7. 35 mm glass cell culture dishes.
8. Humidified tissue culture incubator (37 °C, 5 % CO₂).
9. Falcon polystyrene round bottom tube with cell-strainer cap (BD).
10. Siliconized eppendorf tubes.
11. Cell counter or hemocytometer.
12. Table top centrifuge.
13. 50 ml Falcon tubes.
14. Siliconized dish / surface.
15. Eppendorf GELoader tips (0.5 – 20 µl) (eppendorf,, cat no 5242956003).
16. 12-well tissue culture insert (0.4 µm pore diameter, BD, cat no 353180).
17. 12-well tissue culture plates.

Histogel embedding and manual tissue processing

Reagents

1. 0.5% glutaraldehyde (Sigma, cat no. G7651).
2. PFA (Sigma, P6148).
3. Histogel (Thermo Scientific™, cat no. HG-4000-012).
4. 50 %, 70 %, 94 % and absolute ethanol.
5. Xylene.
6. Wax.

Equipment

1. Water bath.
2. Histology mold.
3. Scalpel.
4. Histology cassette.
5. Warm chamber.

Method details

All procedures – apart from 3.10 and 3.11 – should be carried out in sterile conditions using sterile techniques and instruments. All steps are performed at room temperature unless otherwise specified.

Tooth bioengineering and organ culture

Tissue dissection

1. Keep embryos in PBS on ice in glass petri dishes.
2. Dissect tooth germs (incisors) in PBS using standard procedures (**Fig. 1**). Extra tissues surrounding the tooth germ should be carefully removed.
3. Store tooth germs in serum-free medium on ice in 35 mm glass cell culture dishes.

Note: Serum inhibits dispase activity (Epithelial bud and mesenchymal condensate separation).

Epithelial bud and mesenchymal condensate separation

1. Remove serum-free medium.
2. Immediately add a solution composed of 960 µl Optimem, 40 µl Dispase II and 4 µl DNase I. Make sure tooth germs are submerged into the solution.
3. Incubate for at least 12.5 min at room temperature. Alternatively, incubate for two hours at 4 °C on an agitator.
4. Separate epithelial buds with the help of a fine needle (**Fig. 1**).

Note: The more time, the more efficient the separation.

Epithelial and mesenchymal tissue dissociation

1. Transfer epithelial buds in siliconized eppendorf tubes that contain 0.6 ml of serum-free medium (**Fig. 2**).
2. Do the same for mesenchymal condensates.
3. Let tissues set at the bottom of the tubes.
4. Remove supernatant completely.
5. Add 0.3 ml Accutase in each tube and incubate at 37 °C,
 - a. 20 minutes for epithelial buds and
 - b. 10 min for mesenchymal condensates.
6. Add 0.6 ml of serum-containing medium in each tube.
7. Pipette up and down softly using a 1ml tip, until to see that structures have dissociated into single cells. Epithelial buds are more resistant and require more time.
8. Pass the treated cells through a BD Falcon tube with cell-strainer cap to ensure that all non-single cell aggregates are excluded.

Note: Be careful that only epithelial buds are transferred and no floating mesenchymal cells.

Cell counting and precipitation of single cell suspensions

1. Pipette single cell suspensions into siliconized eppendorf tubes.
2. Resuspend cells.

3. Immediately remove a small aliquot from both the epithelial and the mesenchymal single cell suspensions and count the number of cells by using a cell counter or a hemacytometer.
4. Centrifuge single cell suspensions 5 min at 250-300 x g in room temperature.
5. Remove the supernatant completely without disturbing the pellet. Keep samples on ice.

Note: Use GELoader tips to ensure that no medium has remained. Remaining liquid and / or hampering the cell pellet can result in problems in the injection step.

Bioengineering of germs

1. Prepare Cellmatrix as described in provider's protocol and in *Section 2.2*. Do steps in cold 50 ml falcon tubes that are stored on ice.
2. Let solution to rest on ice for 10 min or until bubbles have disappeared.
3. Make 30 µl droplets on siliconized dish / siliconized surface (**Fig. 3**). Proceed immediately to cell injection.

Cell injection in Cellmatrix droplets

1. Inject 0.2 – 0.3 µl of mesenchymal cells in the center of the Cellmatrix droplet (**Fig. 3**). Use GELoader tips to inject.
2. Inject 0.1 – 0.2 µl of epithelial cells immediately adjacent to the mesenchymal cell aggregate. Use GELoader tips to inject.

Note: Eppendorf GELoader tips they have a long capillary section. Cut to ~ 3 / 4 of the length for better manipulation.

Note: It is important mesenchymal and epithelial cells to be in contact.

Baking of the Cellmatrix droplet

1. Turn the siliconized dish upside down.
2. Incubate drops at 37 °C for 10 min.

Transfer of Cellmatrix droplets in cell culture conditions

Move the Cellmatrix droplet in a 12-well tissue culture insert with the use of forceps. Pay extra attention so the droplets not to be ruptured and cell aggregates disperse from the droplet. Place the droplet so at it touches the bottom of the cell culture insert and preferably not the walls of the cell culture insert. The round drop will be flattened after cultivation, but cells will not move.

Note: Use 6-well tissue culture inserts (and the corresponding tissue culture plates) for greater movement freedom if necessary.

Note: We tried matrigel instead of Cellmatrix. However, at all matrigel concentrations we used, the consistency was not enough to retain the cells in contact.

Organ culture

1. Culture the reconstituted explants for 2 to 14 days on cell culture inserts in 12-well cell culture plates. For 12-well cell culture plates (22.1 mm well diameter) combined with 0.4 μ m pore size inserts (BD, cat no 353180) supplement with 300 μ l / well serum-containing medium per well, or until medium comes into contact with the bottom of the insert (**Fig. 3**).
2. Change the serum-containing medium at a 2 day interval.

*Histogel embedding and manual tissue processing**Histogel encapsulation*

Because of the small size of the reconstituted explants, we suggest that samples are encapsulated in histogel prior to paraffin embedding.

1. Preheat 0.5% glutaraldehyde in 2% PFA (fixative) at 37 °C.
2. Briefly wash the cell culture insert that contains the reconstituted explants twice with PBS.
3. Add fixative so as reconstituted explants are embedded in it (put fixative both on top and bottom of the cell culture insert). Fix for 4 hours with agitation.
4. In the meantime, melt the histogel for >45 min at 60-65 °C water bath.
5. Remove fixative and wash briefly twice with PBS.
6. Take histology molds and place them on ice.
7. Add 100 μ l of histogel on top of the sample (on the top surface of the cell culture insert).
8. Remove the filter from the bottom of the cell culture insert. The reconstituted samples should now be in the histogel.
9. Place reconstituted explants in histology mold.
10. Cover the reconstituted explants with histogel. Start pipetting from the sides and gradually move towards the center to cover the sample. Keep on ice.
11. Wait for histogel to completely solidify. Remove the histogel encapsulated reconstituted explants by scooping it with a scalpel.
12. Place the histogel encapsulated reconstituted explants in a histology cassette and fix with 4% PFA for 1 hour.
13. Briefly wash twice with PBS.

Manual tissue processing

1. Remove the histogel encapsulated reconstituted explants from the histology cassette and put it in a glass vial. Submerge the samples as follows in agitation:
 - a. 50% ethanol for 4 x 30 min.
 - b. 70% ethanol for 4 x 30 min or overnight at 4 °C (change solution to fresh at least four times).

- c. 94% ethanol for 4 x 30 min.
 - d. 100% ethanol for 4 x 30 min.
 - e. Xylene for 4 x 30 min.
2. Remove samples from glass vial and put them on a tissue mold for consequent paraffin embedding.
 3. Put samples in wax for 4 x 30 min (or more) at warm chamber.
 4. Proceed with paraffin embedding.

Note: Be careful not to remove the reconstituted explants along with the filter. In case this happens, use scalpel to carefully remove the reconstituted explants from the filter and put them in the histology mold.

Note: Manual tissue processing is highly recommended as it results in histogel being highly transparent at the end of the process and therefore possible to identify the small tissue.

Journal Pre-proof

Method validation

Using the above protocol, we were able to grow teeth starting from dissociated epithelial and mesenchymal single cell suspensions. Already at day 1, the presumptive teeth primordia are visible (**Fig. 4**). Culture of only epithelial or mesenchymal single cells did not give rise to any identifiable structure (**Fig. 4**). Immunofluorescence of bioengineered teeth shows that cell proliferation is occurring, based on Phospho-Histone3 staining (**Fig. 5**). At day 10, bioengineered teeth have a correct structure, as indicated by hematoxylin/eosin staining (**Fig. 6**). Development of the bioengineered teeth recapitulates *in vivo* development. Importantly, *Sox2*, the dental stem cell marker [14, 15] is upregulated at day 4 (**Fig. 7**).

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

We would like to thank Professor Irma Thesleff, Ms. Riikka Santalahti and Mrs Raija Savolainen for critical discussions and advice. This work was supported by the Academy of Finland.

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Figure legends

Figure 1

Isolation and separation of mesenchymal condensate and epithelial bud from ED14.5 embryos. Incisors are removed, taking special care to remove extra tissues surrounding the tooth germ. Mesenchyme and epithelium are separated by enzymatic treatment, followed by mechanical separation. Scale bars, 1000 μm

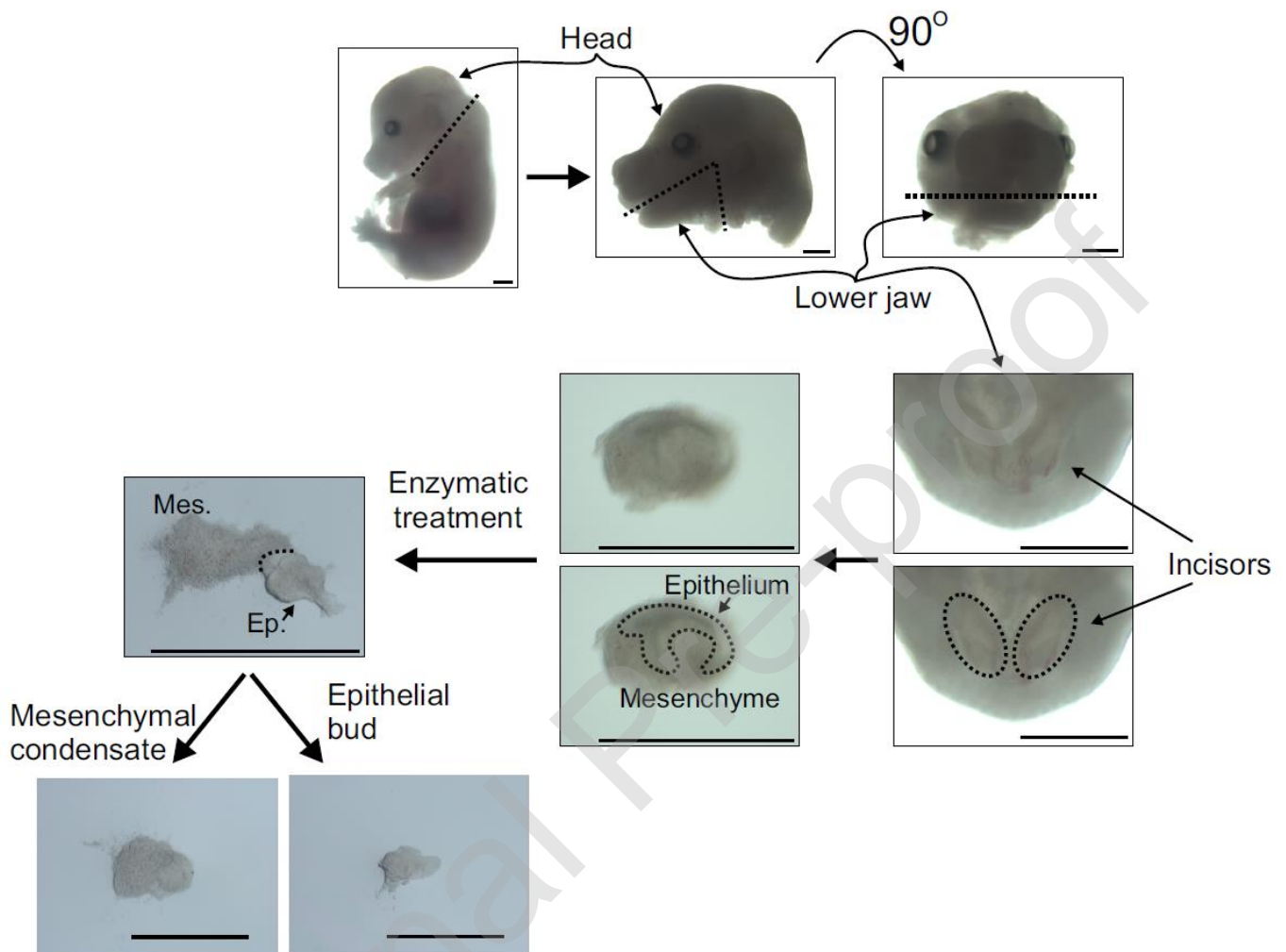


Figure 1_Stratoulis & Michon

Figure 2

Illustration of tissue dissociation and single cell preparation. Epithelial bud and mesenchymal condensate are further treated enzymatically to produce single cell suspensions. In order to ensure that single cells are used in following steps of the protocols, cell suspensions are filtered through a cell strainer.

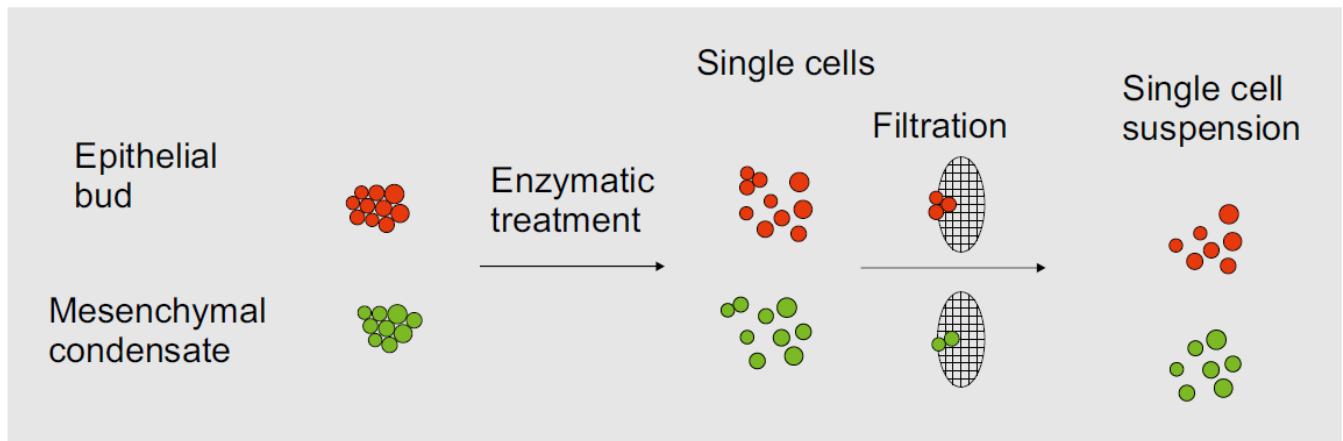


Figure 2_Stratoulas & Michon

Figure 3

Illustration of organ culture preparation. On a siliconized dish, a 30 μ l droplet of Cellmatrix is injected with mesenchymal cells, followed by epithelial cells. Special care should be taken to inject epithelial cells immediately adjacent to the mesenchymal cells. The droplet is transferred on an insert and subsequently cultured.

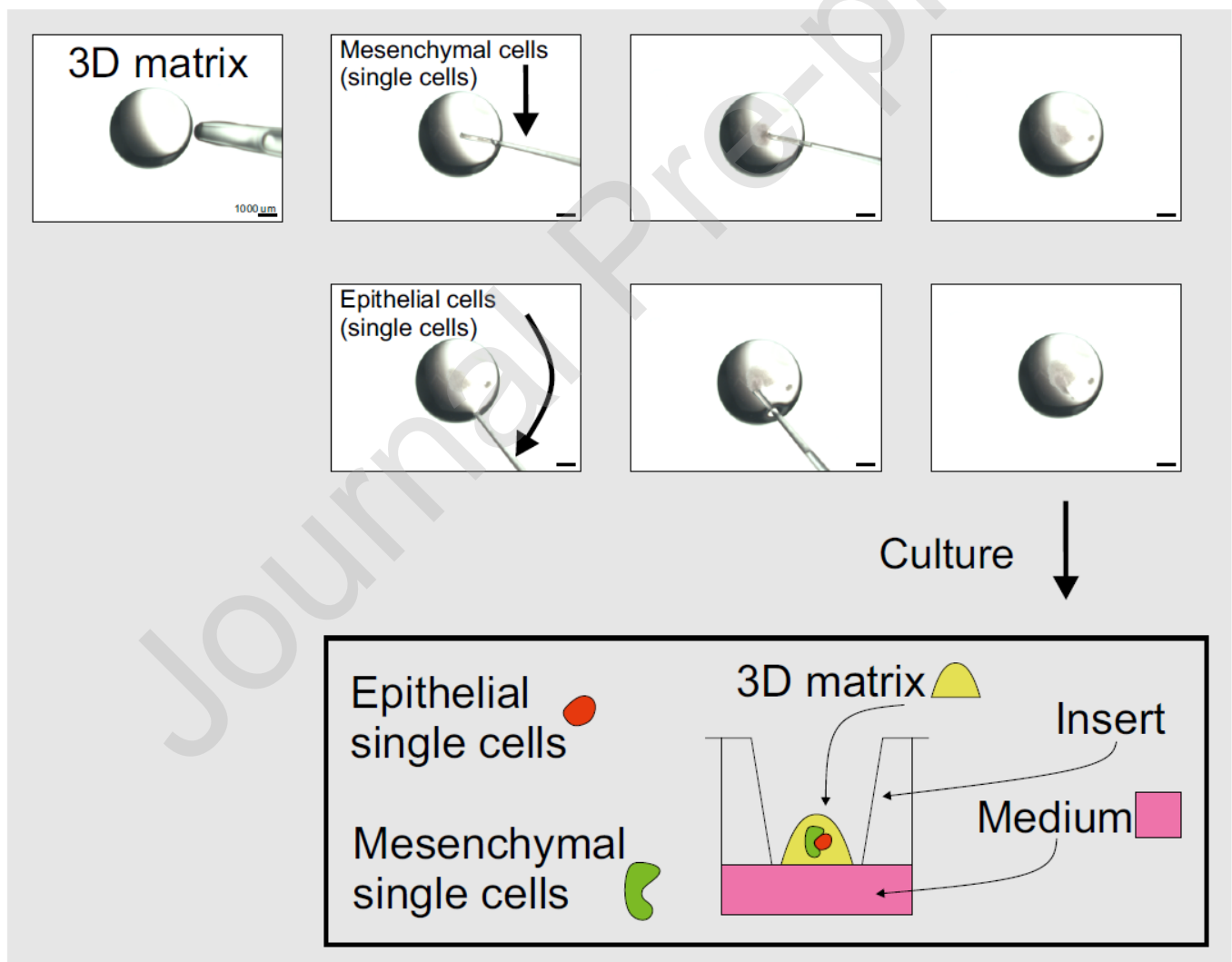
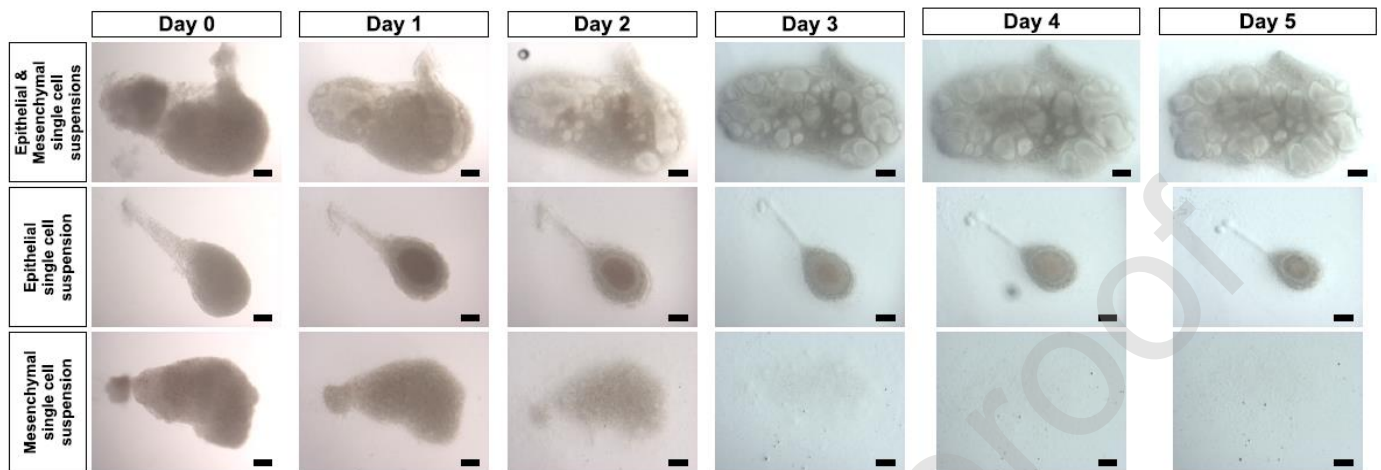


Figure 3_Stratoulas & Michon**Figure 4**

Bioengineered teeth arise from epithelial and mesenchymal single cell suspensions. Mesenchymal or epithelial single cell suspensions on their own are not able to generate any recognizable structure. Scale bars, 200 μ m.

**Figure 4_Stratoulas & Michon****Figure 5**

Hematoxylin/eosin and immunofluorescence stainings on bioengineered teeth sections. At day 4, cells in the bioengineered teeth proliferate, as demonstrated by PhosphoHistone H3 staining (green, ab5176). Tissues were

counterstained with Hoechst 33342 (purple). Black scale bars, 200 μm ; orange scale bars, 50 μm .

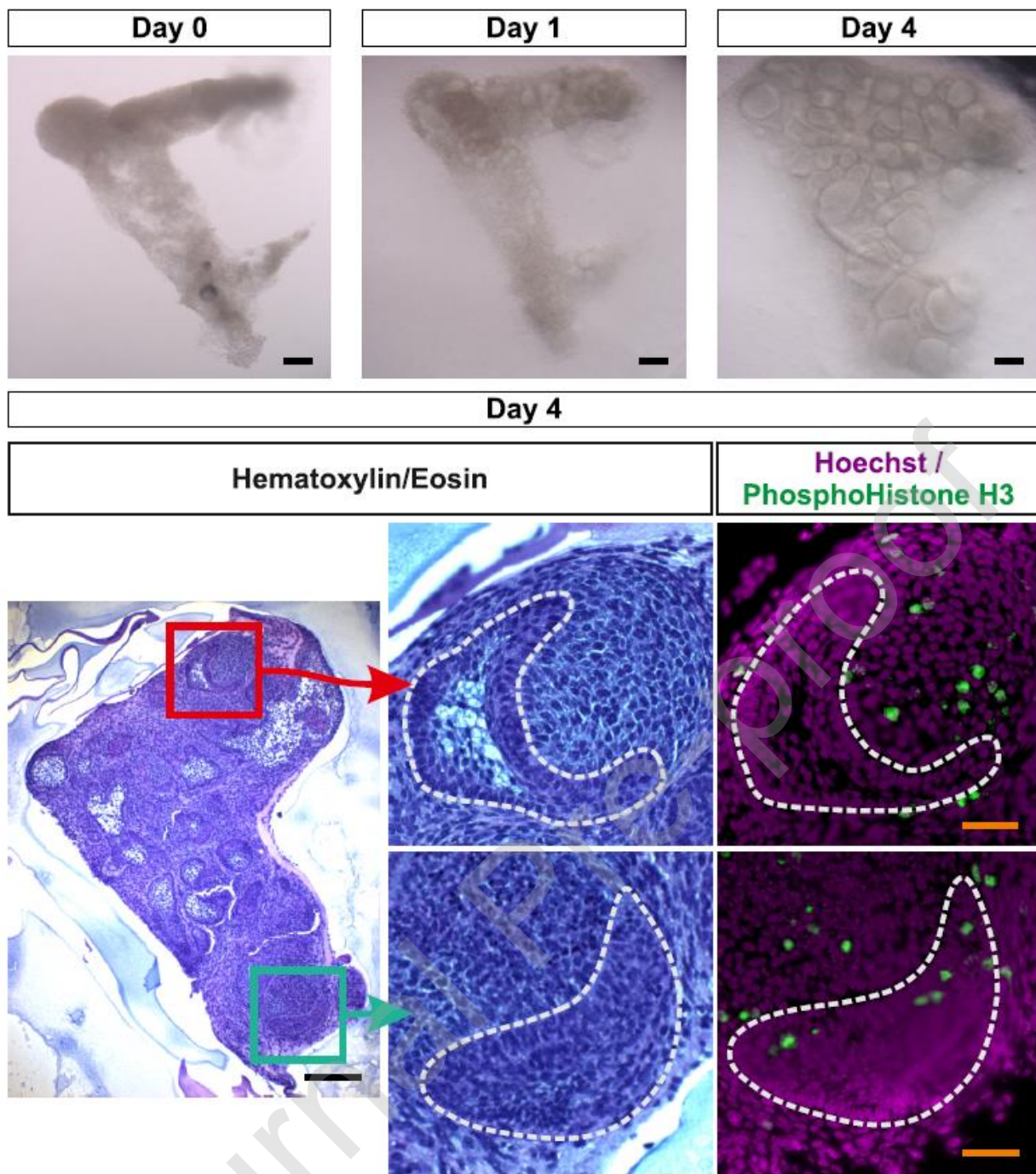


Figure 5_Stratoulas & Michon

Figure 6

Bioengineered teeth are structurally correct. Hematoxylin/eosin staining of ten-day-old bioengineered teeth.

Abbreviations: S.I., Stratum Intermediate; Am, Ameloblasts; E.M., Enamel Matrix; D.P. Dental Pulp. Black scale bars, 200 μm , orange scale bar, 50 μm .

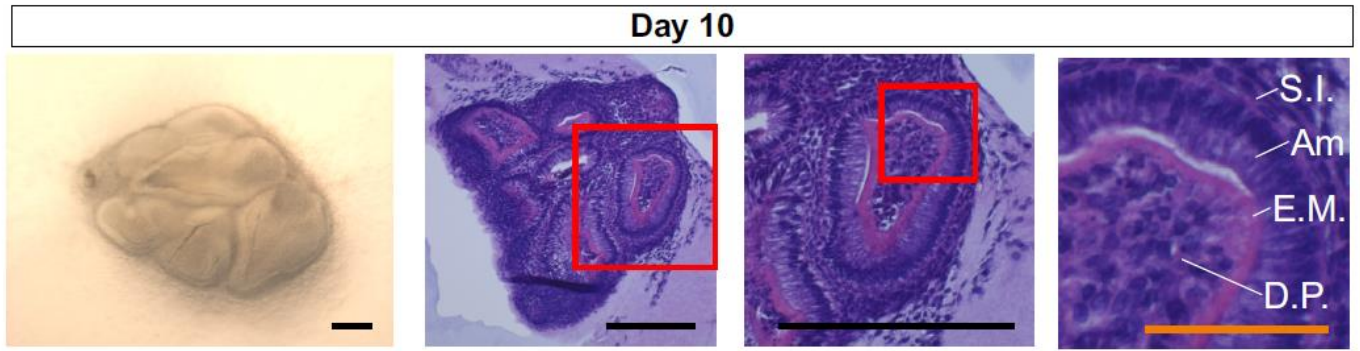


Figure 6_Stratoulis & Michon

Figure 7

Bioengineered teeth express SOX2 already at day 4. Mesenchymal and epithelial cells were isolated from *Sox2-GFP* mice. Scale bars, 200 μ m.

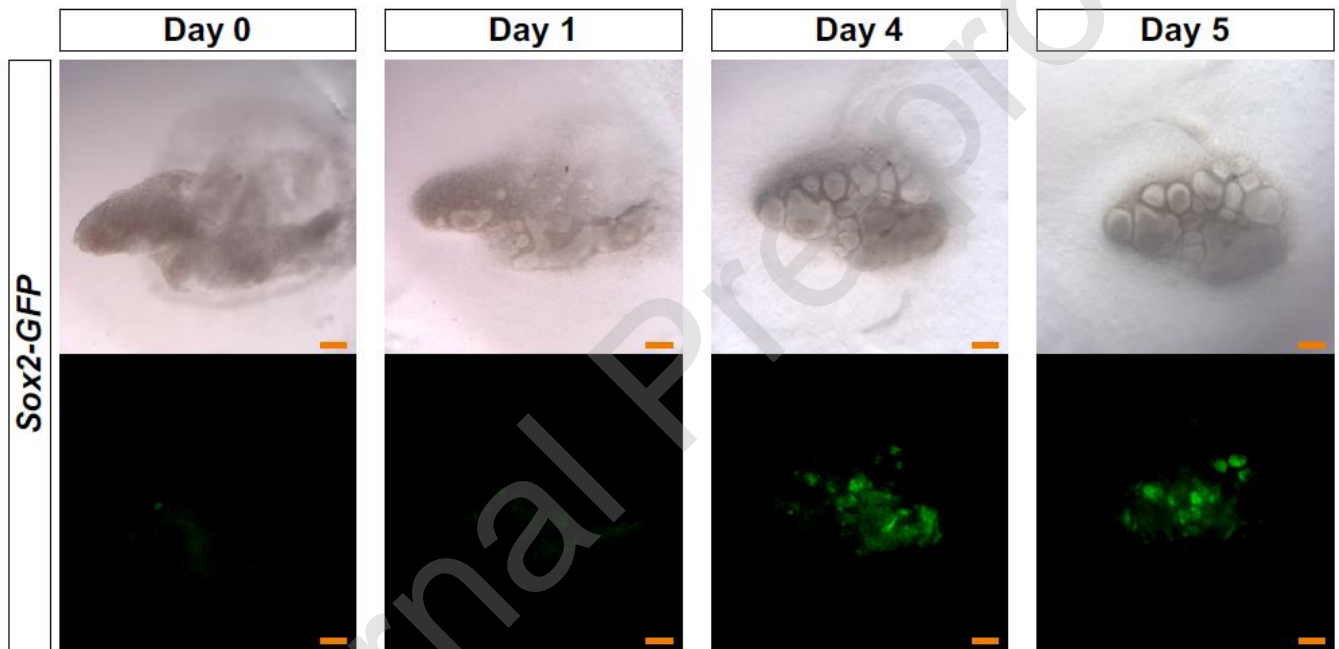


Figure 7_Stratoulis & Michon

SPECIFICATIONS TABLE

Subject Area	<ul style="list-style-type: none"> • <i>Biochemistry, Genetics and Molecular Biology</i> • <i>Medicine and Dentistry</i>
More specific subject area:	<i>Bioengineering</i>
Method name:	<i>Tooth bioengineering</i>
Name and reference of original method	<i>K. Nakao, R. Morita, Y. Saji, K. Ishida, Y. Tomita, M. Ogawa, M. Saitoh, Y. Tomooka, T. Tsuji, The development of a bioengineered organ germ method, Nat Methods 4(3) (2007) 227-30</i>
Resource availability	<i>N/A</i>